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Award Number: W81XWH-11-1-0041

TITLE: Breast Cancer Endothelial Cell Calcium Dynamics Using Two-Photon Microscopy

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REPORT DATE: January 2013

TYPE OF REPORT: Annual Summary

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
January 2013	Annual Summary	1January2012-31December2012

4. TITLE AND SUBTITLE
Breast Cancer Endothelial Cell Calcium Dynamics Using Two-Photon Microscopy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0041

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Rochester
Rochester, NY 14642

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Control murine liver endothelial cells and murine breast tumor endothelial cells were isolated and plated for imaging and flow cytometry experiments. I have found interesting differences between normal and tumor endothelial cells well worth further studying. Specifically, I have found that the response of tumor endothelial cells to VEGF is the same regardless of whether or not they have been serum-starved.

15. SUBJECT TERMS **none provided**

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	11	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction

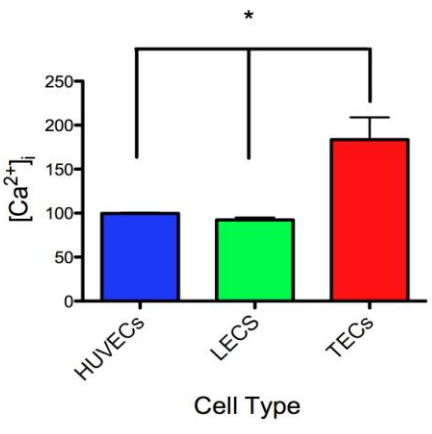
This summary concludes the advances achieved within the second year of this Department of Defense award. The main goal of this project is to study the signaling mechanism downstream of VEGF receptor activation in murine breast adenocarcinoma endothelial cells, which is partly responsible for inducing angiogenic phenotypes in this tumor model. In doing so, I have found interesting differences between normal and tumor endothelial cells well worth further studying. I have grouped my findings into two categories: cells studies under baseline conditions, and experiments that have compared various signal responses between serum-starved and non-serum-starved conditions.

Body

1. Experiments Comparing Tumor and Healthy Endothelial Cells Under Baseline Levels

In addition to extracting endothelial cells from murine mammary adenocarcinomas of TIE2-GFP+ female mice, we initially had proposed to use human umbilical vein endothelial cells (HUVECs) as healthy endothelial cell controls. Due to the technical difficulties of extracting endothelial cells from normal mouse mammary fat pads, I settled for extracting mouse liver microvascular endothelial cells (LECs) and using these normal mouse endothelial cells as a second group of control cells. This way, we have a healthy murine microvascular endothelial cell type to compare, in addition to HUVECs, which have been the gold standard in endothelial cell research. My goal is to repeat all experiments so that the LECs are included as a healthy EC control group for comparison.

Figure 1. The graph shows the intracellular calcium concentrations in HUVECs, LECs, and TECs. Note that there isn't a significant difference in the concentration between the two healthy EC groups; however, the calcium concentration in the TECs analyzed was significantly higher compared to the healthy cell type controls.



We found several key differences between normal and tumor ECs, both qualitative from the appearance of the cells in the experiments, and quantitative, in the degree and extent of the calcium signaling. Upon performing a series of experiments to study these ECs in their baseline environment, we observed that the

intensity of the INDO-1 calcium indicator dye was dimmer in tumor endothelial cells as compared to healthy ECs. Under the same loading conditions, this observation could indicate a difference in their calcium concentrations, and thus we decided to conduct ratiometric measurements of the intracellular calcium levels in these cells. As shown on Figure 1, tumor endothelial cells had a significantly larger concentration of calcium under baseline conditions than the two normal endothelial cell groups analyzed.

Figure 2a. A representative fluorescence trace of a TEC loaded with Indo-1 AM. At the laser excitation wavelength used (750nm) Indo-1 responds to an elevation in free calcium with a decrease in fluorescence, and with an increase in fluorescence when the free calcium concentration decreases. In this case a non-serum-starved TEC (identified by its GFP fluorescence in a separate channel) responds to administration of 1.12μM U73122 to the medium (at the organge arrow) with an increase in calcium.

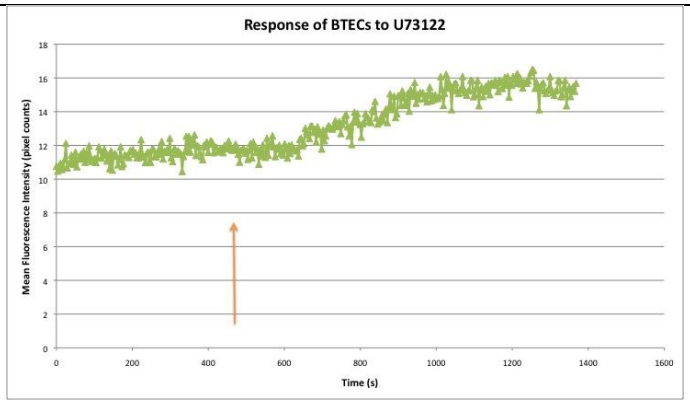
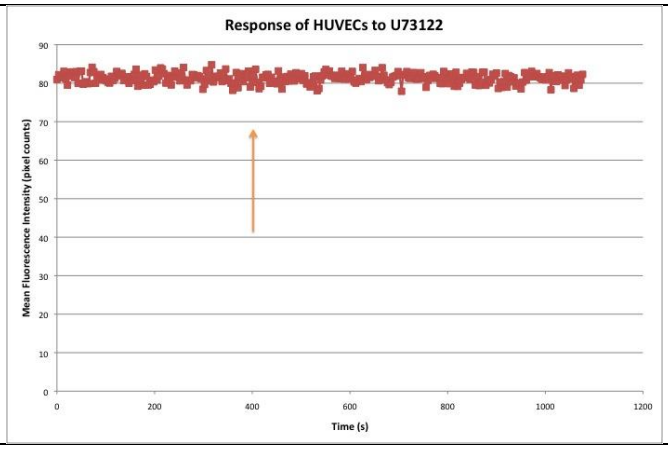
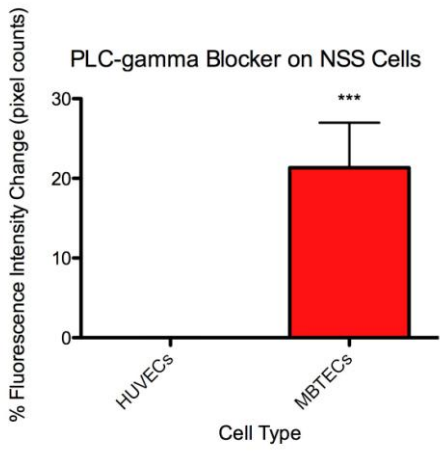


Figure 2b. This image shows a representative fluorescence trace of a HUVEC from an experiment in which, in the same way as Figure 2a, U73122 was applied over a period of time. Note how the fluorescence doesn't change over time.



Tumor ECs having a larger concentration of calcium than healthy ECs could mean that there is a constitutively active VEGF signaling mechanism that might be responsible for keeping the calcium concentration in the cytosol higher than otherwise. The next set of experiments involved trying to cut off the VEGF signaling pathway by blocking PLC-γ with U73122 and observing whether the calcium levels in tumor ECs changes. Upon application of 1.12uM of U73122 to HUVECs and tumor ECs we observed a significant increase in mean percent fluorescence intensity change in the tumor EC population, as opposed to almost no change at all for the

normal ECs, confirming our predictions that part of the VEGF signaling mechanism in tumor ECs must be constantly active (Figures 2 and 3).

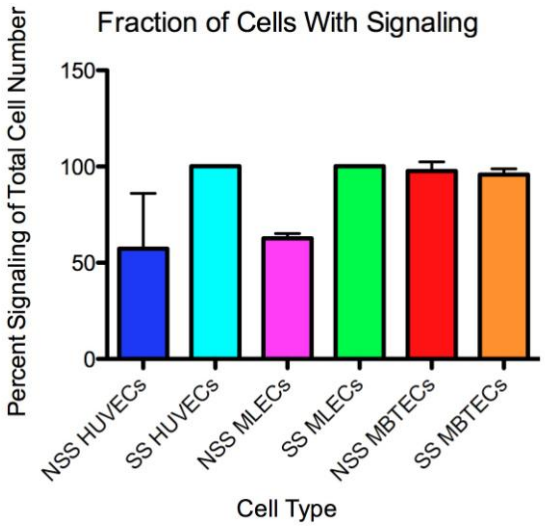
Figure 3. Data from the application of U73122 were pooled and the percent change in fluorescence intensities were analyzed between NSS HUVECs and NSS TECs.	

2. Experiments Comparing Serum-Starved vs. Non-Serum-Starved Conditions

Given that we have observed interesting differences in the VEGF signaling mechanism between healthy and tumor ECs at baseline levels with their respective GF-rich media, and furthermore that the density of their VEGFRs is different, we wanted to find out whether there would be differences in how these cell groups respond to a step function of more VEGF into the system when they're both SS and NSS.

In order to analyze single cells from our imaging experiments using the two-photon microscope, we came up with a quantitative rationale to determine whether a cell has signaled after the addition of the VEGF solution. If the percent fluorescence intensity change for a single cell is equal to or greater than 5% from the average baseline fluorescence intensity level, the cell is considered as having signaled and is used in the analysis for comparison with other cell types and conditions. Otherwise, it is considered as a cell that did not signal. The graph on Figure 4 shows the percentage of cells in each group with a mean percent change in fluorescence intensity over 5% from baseline. Note how both normal EC groups show significantly lower signaling when NSS but both NSS and SS TEC groups have the same level of signaling of nearly 100% of the cells analyzed.

Figure 4. Percent signaling in cells analyzed. These bars show the percentage of cells that had fluorescence intensity changes of 5% or higher from average baseline fluorescence intensity. It is important to note that usable baselines had a percent change in fluorescence intensity of less than 2.5%.



Our most interesting results so far are VEGF-signaling differences between normal endothelial cells and TECs. Typically, endothelial cells undergo 24 hours of serum starvation as is often done to enhance healthy EC response to VEGF (serum itself contains VEGF). Our results show that there is no significant difference between the signaling in TECs for the serum-starved and the non-serum-starved conditions, which is not the case with HUVECs and LECs. The graphs in figures 5 and 6 compare the percent change in fluorescence intensity levels among the cells in each group that had signaling higher than 5% from baseline fluorescence levels. On both graphs we observe two very interesting results – NSS and SS TECs have the same intensity change and their level are significantly different than both SS and NSS normal EC groups. Data was analyzed using one-variable ANOVAs with Bonferroni Correlation post-hoc tests to analyze significance between each pair of data groups.

Figure 5. Individually analyzed ECs from two-photon fluorescence intensity data were grouped into non-serum-starved (NSS) HUVECs, serum-starved (SS) HUVECs, NSS TECs, and SS TECs. This figure shows cells that signaled analyzed by their percent change mean fluorescence intensity values. ($p<0.0005$)

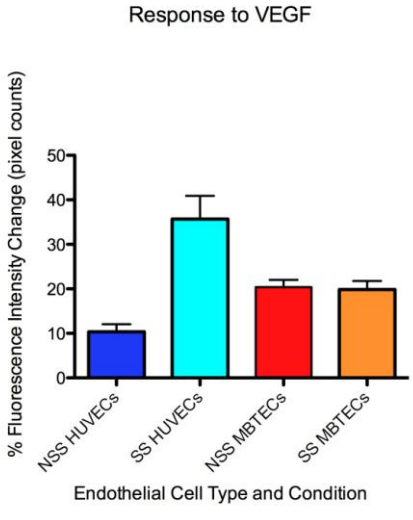
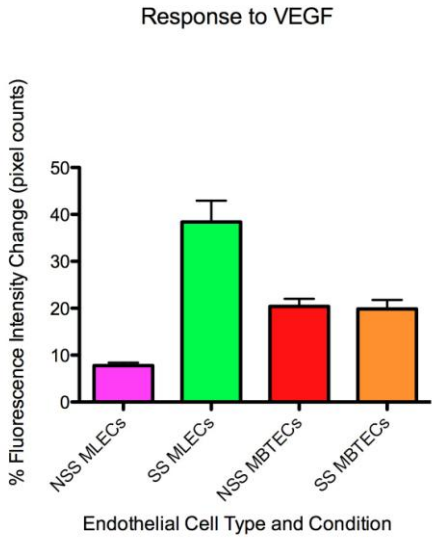


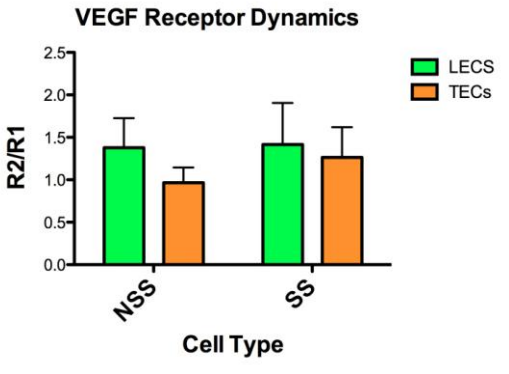
Figure 6. This figure shows similar results to the ones shown in Figure 5, but the control group is the mouse live endothelial cell group. Signaling differences between both serum-starved and non-serum starved TECs and the non-serum starved LECs were found. ($p<0.0005$)



A difference in signaling between tumor and healthy ECs could be explained by either a difference in the way these cell groups use their receptor manipulation mechanisms in order to alter response to VEGF, or it could mean that one or more downstream signaling player is accounting for the differences observed. Given the strong evidence that the VEGF signaling mechanism in tumor ECs was fundamentally different from the normal ECs, I sought to investigate if the differences observed in the two-photon imaging experiments could be explained somehow by looking into the density of VEGF receptors (VEGFRs), VEGFR-1 and -2, both of which have different binding affinities to VEGF and signaling strength.

I then performed at least three flow cytometry experiments for each EC line with the NSS and the SS conditions and tagging for R1 and R2. When I analyzed the data I decided to compare the ratios of R2/R1 since this number would indicate the strength of the signaling response given what we know about these two VEGFRs. A two-way ANOVA indicated that there is no significant contribution of the serum-starvation in the change in receptor ratio, nor does there exist a significant difference between the tumor and healthy endothelial cells in the way they alter their receptors (Figure 7).

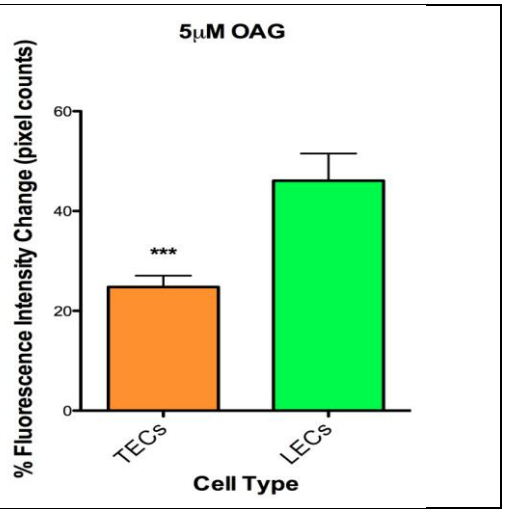
Figure 7. Receptor densities were compared between liver ECs (green) and tumor ECs (orange). The y-axis represents the ration of VEGFR-2/VEGFR-1 given that VEGFR-1 is known as a VEGF trap with much higher affinity for VEGF but a lower signaling capacity than VEGFR-2. A two-way ANOVA analyzing the effects of serum-starvation and the difference in cell lines determined there were no significant effects of either factor.



Now, since we have identified a key difference in the way tumor endothelial cells respond to an addition of VEGF and we know from our flow cytometry experiments that their receptor densities are different, we wanted to find out if we would obtain the same response that we obtained when we added VEGF by bypassing the receptors and PLC-gamma. We accomplished this by adding OAG, which is a soluble form of DAG. Initially we started out with a high concentration of the reagent that has been the norm in previous studies. However, when we compared the intensity change signals with the change in signals obtained from the VEGF application experiments, we realized that the signals were almost two-fold higher and were unable to determine any differences between the groups. This meant we were saturating our system and had to test lower concentrations. We performed dose response curves with the healthy ECs and determined the ideal concentration to be 5uM of OAG.

In Figure 8 we can observe the data summary that compares LECs and TECs upon application of 5um OAG.

Figure 8. Results from OAG experiments after determining the ideal concentration of OAG (5uM). This figure compares NSS LECs and TECs. The percent change in fluorescence intensity is significantly lower in TECs groups as compared to the healthy ECs.



Our results indicate the percentage change in fluorescence intensity for TECs group is significantly lower than the LECs group, suggesting that TECs might have either a lower response for OAG or they might have a different level of TRPC receptors.

Key Research Accomplishments in the Past Year

1) Obtained statistically significant differences between normal and endothelial cells under their baseline environments, and between their serum-starved and non-serum starved states.
2) Found key VEFG-signaling and OAG-induced-signaling differences between normal endothelial cells and tumor endothelial cells.
3) Determined that the relative surface receptor densities for VEGFR-1 and VEGFR-2 do not vary significantly between healthy and tumor populations, and furthermore that they do not change significantly between the serum-starved and non-serum starved case.

Reportable Outcomes

Over the past year I have attended the following conferences with the funds from this award:

American Association for Cancer Research Annual Meeting, Chicago, IL, April 2012
Biomedical Engineering Society Annual Meeting, Atlanta, GA, October 2012

I have presented my work through the following oral presentation:

Lapeira-Soto J, Madden KS, Brown EB, Multiphoton Microscopy Reveals Abnormal Ca^{2+} Signaling in Breast Tumor Endothelial Cells, Biomedical Engineering Society Annual Meeting, 2012

I have also presented my work as poster presentations as follows:

Lapeira-Soto J, Madden KS, Brown EB, Abnormal VEGF-induced Calcium Signaling in Purified Breast Tumor Endothelial Cells, American Association for Cancer Research Annual Meeting, 2012

Lapeira-Soto J, O'Connell P, Perry S, Brown EB, Brown E, Expanding the applicability of multiphoton fluorescence after photobleaching by incorporating shear stress in a laminar flow model, Biomedical Engineering Society Annual Meeting, 2012

As part of my training plan, in the past year I have attended various seminars and discussed topics of relevance pertaining to breast cancer with researchers at the medical center.

Conclusion

I have concluded the second year of my project in which I have demonstrate that there are significant differences in signaling between healthy and breast tumor endothelial cells. The work I have performed so far has involved aspects detailed in Aim 1 and Aim 2 detailed in my Statement of Work and I have following the items I proposed in my training plan. The imaging studies performed *in vitro* laid the groundwork to start investigating these results in vivo, as was proposed in my research plan. I had previously determined that the fact there is no significant difference in signaling capacity between the serum-starved and non-serum-starved tumor endothelial groups signifies that this project is worth translating into an *in vivo* setting. This year I have determined that molecular players downstream of VEGF receptors are significantly different in tumor endothelial cells as compared to the healthy controls. These findings make an interesting case for the possibility of finding intracellular signaling players that could represent the convergence of various angiogenic signaling pathways in tumor endothelial cells.